STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF METABOLIC PROTEINS FROM *LEISHMANIA DONOVANI* AS POTENTIAL DRUG TARGETS

A THESIS SUBMITTED TO

INSTITUTE OF ADVANCED RESEARCH

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY (Ph.D.)
IN
BIOTECHNOLOGY

BY

BHUMI PATEL

UNDER THE GUIDANCE OF

DR. REENA AGRAWAL-RAJPUT

&

DR. ANJU PAPPACHAN

DEPARTMENT OF BIOLOGICAL SCIENCES & BIOTECHNOLOGY
INSTITUTE OF ADVANCED RESEARCH
GANDHINAGAR-382426, GUJARAT, INDIA

JULY 2019

Table of Contents

	Page Nos.
Declaration	III
Certificate	IV
Acknowledgments	V
Abstract	VII
Abbreviations	XV
List of Figures	XVIII
List of Tables	XXI

Chapter 1:	**Introduction**		1
1.1.	*Leishmania*		2
	1.1.1.	Taxonomy	2
	1.1.2.	Morphology	2
	1.1.3.	Life cycle	3
	1.1.4.	*Leishmania* genome	4
1.2.	Leishmaniasis		5
	1.2.1.	Cutaneous leishmaniasis (CL)	6
	1.2.2.	Mucocutaneous leishmaniasis (MCL)	7
	1.2.3.	Visceral Leishmaniasis (VL)	7
	1.2.4.	Disease transmission vectors	8
	1.2.5.	Diagnosis and treatment	9
1.3.	Identification of drug targets in *Leishmania*		11
1.4.	Potential drug targets in *Leishmania*		11
	1.4.1.	Purine salvage pathway	12
		1.4.1.1. Phosphoribosyl transferases	13
		1.4.1.2. Xanthine phosphoribosyl transferase	16
	1.4.2.	Carbohydrate metabolism pathways	16
		1.4.2.1. Hexokinase	18
		1.4.2.2. Glucokinase	20

1.5.	Rationale behind the current study		21
1.6.	Aims and objectives of the present study		22
Chapter 2:	**Structural and functional characterization of Xanthine phosphoribosyl transferase (LdXPRT) enzyme from *L. donovani*.**		23
2.1.	Introduction		24
2.2.	Materials		29
	2.2.1.	Chemicals and reagents	29
	2.2.2.	Genomic DNA and primers	29
	2.2.3.	Cloning vectors and strain	30
2.3.	Methods		30
	2.3.1.	Gene amplification and cloning of LdXPRT	30
	2.3.2.	Expression of LdXPRT in *E. coli* expression strains	31
	2.3.3.	Purification of recombinant LdXPRT using affinity chromatography	32
	2.3.4.	Validation of purified LdXPRT using MALDI-TOF MS analysis	32
	2.3.5.	Molecular mass determination	32
	2.3.6.	Structural characterization 2.3.6.1. Crystallization and screening 2.3.6.2. Homology modeling and structure validation	33 33 33
	2.3.7.	Bioinformatics analysis 2.3.7.1. Sequence analysis and phylogeny 2.3.7.2. Comparison of LdXPRT with its nearest homolog in human (HGPRT)	34 34 34
	2.3.8.	Functional characterization of LdXPRT by enzyme kinetic assay	35

	2.3.9.	Screening of inhibitor compounds for LdXPRT	36
		2.3.9.1. Virtual screening	36
		2.3.9.2. Enzyme inhibition assay	36
		2.3.9.3. Inhibitor binding analysis of LdXPRT by fluorescence spectroscopy	37
		2.3.9.4. Inhibitor binding analysis of LdXPRT by CD spectroscopy	38
2.4.	Results		39
	2.4.1.	PCR amplification and cloning	39
	2.4.2.	Expression and purification of recombinant LdXPRT	40
	2.4.3.	Validation of purified protein using MALDI-TOF MS analysis	42
	2.4.4.	Gel filtration chromatography	43
	2.4.5.	Structural characterization	44
		2.4.5.1. Crystallization and screening	44
		2.4.5.2. Homology modeling and structure validation	45
	2.4.6.	Bioinformatics analysis	48
		2.4.6.1. Multiple sequence alignment	48
		2.4.6.2. Phylogeny	49
		2.4.6.3. MEME analysis for motif identification	51
		2.4.6.4. Comparison of the active site of LdXPRT with its nearest human homolog (HGPRT)	52
		2.4.6.5. Comparison of secondary structure pattern and C-alpha positions of LdXPRT and its nearest human homolog (HGPRT)	54
	2.4.7.	Functional characterization of LdXPRT	55
		2.4.7.1. Enzyme activity and pH dependence	55
		2.4.7.2. Enzyme kinetic analysis	56

	2.4.8.	Screening of inhibitor compounds for LdXPRT	56
		2.4.8.1. Virtual screening	56
		2.4.8.2. Enzyme inhibition assay	62
		a) Percent inhibitory activity	63
		b) Inhibition kinetics	64
		2.4.8.3. Inhibitor binding analysis of LdXPRT by fluorescence spectroscopy	67
		2.4.8.4. Inhibitor binding analysis of LdXPRT by CD spectroscopy	68
		a) Secondary structural aspects	69
		b) Tertiary structural aspects	69
		c) Thermal denaturation curve	69
2.5.	Discussion		72
Chapter 3:	**Characterization of LdXPRT mutants to identify the molecular determinants of its substrate specificity**		76
3.1.	Introduction		77
3.2.	Materials		78
3.3.	Methods		78
	3.3.1.	Bioinformatics analysis	78
	3.3.2.	Cloning of LdXPRT mutants	78
	3.3.3.	Expression and purification of LdXPRT mutants	80
	3.3.4.	Structural characterization of LdXPRT mutants using CD and UV absorption spectroscopy	81
	3.3.5.	Functional characterization of LdXPRT mutants	81
		3.3.5.1. Fluorescence spectroscopy	81
		3.3.5.2. Enzyme kinetic analysis	82
3.4.	Results		82
	3.4.1.	Bioinformatics analysis	82
	3.4.2.	Cloning of LdXPRT mutants	84
	3.4.3.	Expression and purification of LdXPRT mutants	89

	3.4.4.	Structural characterization of LdXPRT mutants using CD and UV absorption spectroscopy	90
	3.4.5.	Functional characterization of LdXPRT mutants 3.4.5.1. Fluorescence spectroscopy 3.4.5.2. Enzyme kinetic analysis	95 95 98
3.5.	Discussion		99
Chapter 4:	**Structural and functional characterization of Hexokinase (LdHK) and Glucokinase (LdGK) from *L. donovani*.**		104
4.1.	Introduction		105
4.2.	Materials		105
	4.2.1.	Chemicals and reagents	105
	4.2.2.	Genomic DNA and primers	106
	4.2.3.	Cloning vectors and strain	106
4.3.	Methods		107
	4.3.1.	Gene amplification and cloning 4.3.1.1. Cloning of LdHK 4.3.1.2. Cloning of LdGK	107 107 108
	4.3.2.	Expression trials in *E. coli* expression strains 4.3.2.1. Expression of LdHK 4.3.2.2. Expression of LdGK	109 109 109
	4.3.3.	Purification of recombinant LdGK using affinity chromatography	110
	4.3.4.	Validation of LdGK using MALDI-TOF MS analysis	110
	4.3.5.	Molecular mass determination	110
	4.3.6.	Structural characterization of LdGK 4.3.6.1. Crystallization and screening 4.3.6.2. CD spectroscopy 4.3.6.3. Homology modeling and structure validation	110 110 111 111
	4.3.7.	Bioinformatics analysis of LdGK 4.3.7.1. Sequence analysis and phylogeny 4.3.7.2. Comparison of LdGK with human glucokinase	111 111 112

	4.3.8.	Screening of inhibitor compounds for LdGK by Docking	112
4.4.	Results		113
	4.4.1.	Gene amplification and cloning	113
		4.4.1.1. Gene amplification and cloning of LdHK	113
		4.4.1.2. Gene amplification and cloning of LdGK	116
	4.4.2.	Expression and purification of recombinant proteins	117
		4.4.2.1. Expression trials of LdHK	117
		4.4.2.2. Expression and purification of LdGK	119
	4.4.3.	Validation of purified LdGK using MALDI-TOF MS analysis	121
	4.4.4.	Gel filtration chromatography of LdGK	122
	4.4.5.	Structural characterization LdGK	122
		4.4.5.1. Crystallization and screening	122
		4.4.5.2. CD spectroscopy	125
		4.4.5.3. Homology modeling and structure validation	126
	4.4.6.	Bioinformatics analysis LdGK	127
		4.4.6.1. Sequence analysis and phylogeny	127
		4.4.6.2. Comparison of LdGK with human glucokinase	131
	4.4.7.	Screening of inhibitor compounds for LdGK by Docking	133
4.5.	Discussion		135
Chapter 5:	**Summary and Conclusions**		138
References			145
List of Publications, Conference presentations, Workshops			154

Abstract

Leishmania are protozoan parasites causing spectra of diseases ranging from cutaneous lesions to debilitating mucocutaneous and lethal visceral infections. Leishmaniasis is prevalent in many tropical and subtropical countries and is found in parts of about 88 countries. Visceral leishmaniasis (kala-azar) which is caused by *L. donovani* is the second-largest parasitic killer in the world (after malaria), responsible for an estimated 500,000 infections each year worldwide**.** The treatment of leishmaniasis is difficult, because of the presence of three different forms and various clinical manifestations. High toxicity, high cost and the emergence of drug resistance is a major concern with the existing leishmanial drugs. Hence, there is a need to identify and characterize the new prospective drug targets and to develop parasite-specific inhibitors.

Thus, in the present study, we selected three enzymes (xanthine phosphoribosyl transferase (LdXPRT), hexokinase (LdHK) and glucokinase (LdGK)) from the purine salvage pathway and carbohydrate metabolism pathway of *L. donovani* to characterize their structural and functional aspects to exploit them for selective inhibition. The target proteins LdXPRT and LdGK were cloned, expressed and purified successfully. The LdHK was insoluble hence it was not purified. The XPRT was not present in other protozoan parasites and humans. LdGK presents close ancestral association with the glucokinases from *Trypanosoma* and *Trichomonas vaginalis* however, it was evolutionarily very distant from human glucokinase.

The crystallization trials were carried out to obtain three-dimensional structures of the target proteins. Most of the crystals conditions gave salt crystals while few were not suitable for diffraction by X-ray beam hence computational approach (Homology modeling) was applied to obtain the structure of target proteins. The LdXPRT model is an α/β type protein carrying the PRTase type I folding pattern which has a characteristic core domain consisting of phosphate binding site and the hood domain consisting of purine binding site. The comparison of the LdXPRT and glucokinase LdGK models with the human counterpart reveals significant differences which can be utilized for designing of potential parasite-specific inhibitors. The enzyme assay showed that the LdXPRT can recognize hypoxanthine and guanine, in addition to its natural substrate xanthine.

To identify the potential inhibitors for these proteins, the multidisciplinary approach was used right from the computational screening of compounds to identify the affinity and inhibitory activities of the compounds by biochemical & biophysical approach. The compound libraries of substrate analogs, existing antileishmanial drugs and natural compounds were screened against LdXPRT and LdGK by virtual screening. Few compounds from docking studies of LdXPRT were characterized further for their affinity and inhibitory potential by fluorescence spectroscopy and enzyme inhibition assay. Four (dGMP, cGMP, dGDP & GTP) compounds showed competitive inhibition with both the substrates (xanthine & PRPP) of LdXPRT. Binding of inhibitors causes a minor shift in the secondary structure of LdXPRT with a noticeable change in tertiary structure pattern. Similarly, LdGK inhibitors were also identified by virtual screening. Two inhibitor compounds (dGDP and cGMP) were identified which can probably inhibit both LdXPRT and HGPRT enzymes of the purine salvage pathway. Further optimization of these compounds can give potential drug molecules against leishmaniasis.

Sequence and structure comparison of LdXPRT with *L. tarentolae* HGPRT identified three probable amino acids (Ile 209, Glu 215 and Tyr 208) that may constitute the basis for purine base specificity of the LdXPRT. Mutation analysis of these amino acids was carried out to probe their role is substrate specificity. A 28 residue long unique motif was identified in LdXPRT, which was absent in other PRTases including human and leishmanial HGPRTs. This region gives a higher fluctuation in MD simulations upon binding with the substrate. Hence to investigate the role of this region in XPRT activity and/or specificity, this region was deleted from LdXPRT for further characterization. The secondary and tertiary structure pattern of LdXPRT and ldxprtY208F were almost similar although other mutants have some differences from the wild type protein. The I209V and I209V;E215D mutations converted LdXPRT to HGXPRT which exhibits nucleobase specificity comparable to *T. gondi, T. foetus* and *P. falciparum* HGXPRT. Mutational studies showed that the presence of Ile at position 209 in the purine binding pocket might be responsible for the substrate specificity of the LdXPRT. Deletion of unique motif L55_Y82del ceases the activity of the enzyme indicating that the region is either important for enzyme activity or it might be altering the enzyme's structure in such a way that it could not perform enzyme reaction.

Chapter 1

Introduction

1.1. *Leishmania*

Leishmania is a protozoan parasite capable of causing infection in a variety of animals including hyraxes, canids, rodents, and humans. *Leishmania* parasites are named after a British army surgeon Sir William Boog Leishman. These parasites are found in all continents except Antarctica (Bañuls et al., 2007). Out of the 53 species of the parasite, 20 species are pathogenic for humans and 31 species are pathogenic for other mammals. The *Leishmania* is categorized into two groups. The species which are prevalent in Asia, Africa and Europe are categorized as old world species and the species which are found in America are called new world species (Alemayehu and Alemayehu, 2017).

1.1.1. Taxonomy

The genus *Leishmania* is classified in two subgenera depending on their distribution in the gut of sandfly vector. Parasites which colonize in the midgut and foregut of sandfly are grouped as the genus *Leishmania* whereas the parasites which develop in hindgut are called *Viannia*. The subgenus *Leishmania* is further classified into *L. donovani* complex, *L. major, L. tropica, L. aethiopica,* and *L. mexicana* complex. Whereas *Viannia* is classified into *L. braziliensis* complex, *L. anensis, L. naiffi* and *L. lainsoni* complex. The systematic classification (Akhoundi et al., 2016; Bañuls et al., 2007; Maurício, 2018) of *Leishmania* is given as follows.

Kingdom:	Protista
Subkingdom:	Protozoa
Phylum:	Euglenozoa
Class:	Kintoplastida
Subclass:	Metakinetoplastina
Order:	Trypanosomatida
Family:	Trypanosomatidae
Genus:	Leishmania

1.1.2. Morphology

Leishmania exists in two different morphological forms depending on the host. When present in sandfly midgut, it exists as elongated, flagellated and motile promastigote form

(Figure 1.1 A). Promastigotes are spindle-shaped with 15-20 µm length & 1.5-3.5 µm width. These Promastigotes form are introduced into the mammals by the bite of the sandfly. Upon entry into host macrophage, promastigotes transform into amastigotes inside the macrophages. Amastigotes are non-flagellated, non-motile, spherical structures of size 2-4 µm in diameter (Figure 1.1 B) (Herwaldt, 1999). Instead of having a flagellum, amastigotes have a delicate filament which does not expand out of the body. Its flagellar pocket acts as a place for endocytosis and exocytosis. *Leishmania* has a well-defined nucleus and the kinetoplast. The cytoplasm of *Leishmania* is vacuolated.

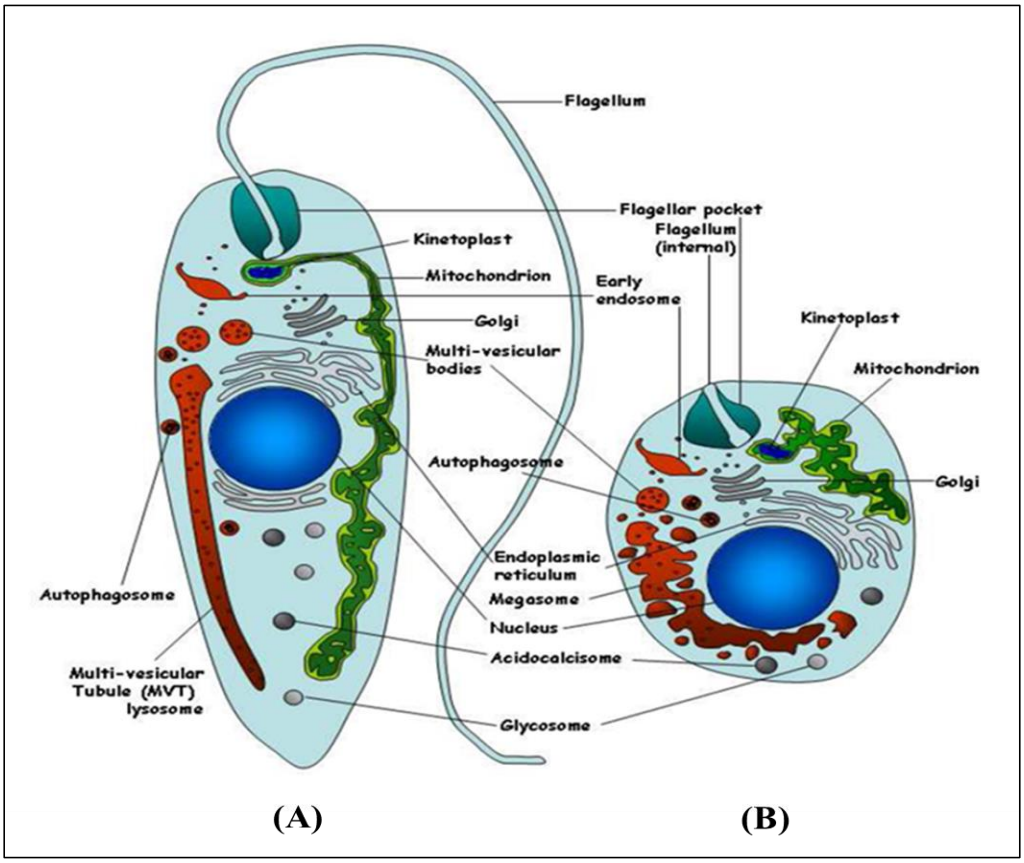


Figure 1.1: Structure of *Leishmania* parasite. (A) Promastigote form; (B) Amastigote form. Image source: (Besteiro et al., 2007).

1.1.3. Life cycle

Leishmania has a dimorphic life cycle which partially progresses in mammals (primary reservoirs) and partially in sand flies (secondary hosts) (Figure 1.2). The cycle starts when the infected sand fly bites to its host where the promastigotes are ingested into the

host body. Promastigotes are then taken up by the host macrophages and get converted to amastigote form which further multiply by binary fission. Eventually, the macrophages burst and release the amastigotes into the bloodstream. These amastigotes further infect other organs and likewise, the infection spreads. The amastigotes are transferred in the sandflies by ingesting infected cells during blood meals. The amastigotes then transform into promastigote form in the hindgut (in case of subgenus *Leishmania*) or midgut (in case of subgenus *Viannia*) of sand fly (Farrell, 2002). The promastigotes then multiply by binary fission. This stage is avirulent. After 4 to 5 days of feeding, the promastigotes move towards the esophagus and salivary glands of the fly and become virulent. This process is called metacyclogenesis (Sacks, 1989). These virulent forms of promastigotes are ingested into the mammalian host during their next feed.

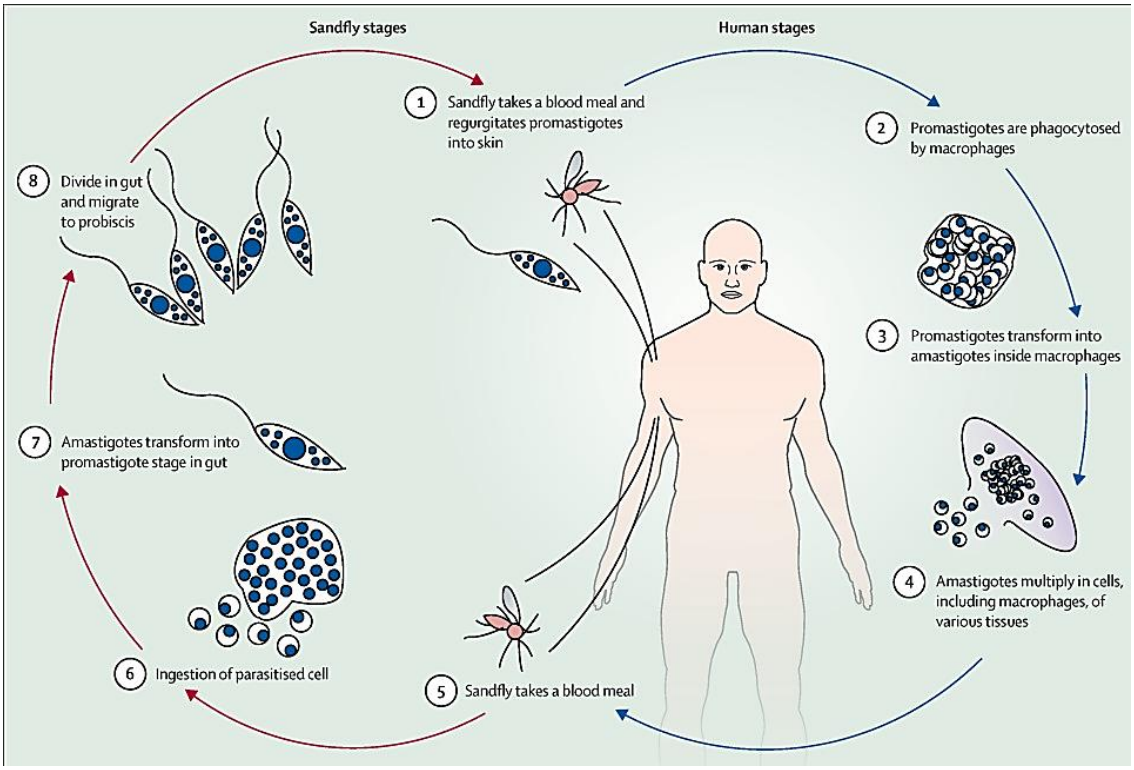

Sandfly stages
Human stages
1 Sandfly takes a blood meal and regurgitates promastigotes into skin
2 Promastigotes are phagocytosed by macrophages
3 Promastigotes transform into amastigotes inside macrophages
4 Amastigotes multiply in cells, including macrophages, of various tissues
5 Sandfly takes a blood meal
6 Ingestion of parasitised cell
7 Amastigotes transform into promastigote stage in gut
8 Divide in gut and migrate to probiscis

Figure 1.2: Life cycle of *Leishmania*. Image source: (Burza et al., 2018)**.**

1.1.4. *Leishmania* genome

The *Leishmania* genome is organized in the nucleus, containing chromosomal and episomal DNA, as well as in the kinetoplasts (modified mitochondria), which includes the independently replicating DNA molecules. In contrast to other Trypanosomatidae

members, *Leishmania* genome lacks the sub-telomeric region and the transposable element. The old world *Leishmania species* have 36 chromosomes, whereas the new world species have 34 or 35 chromosomes.

In 2005, the first whole-genome sequencing project of *Leishmania* parasite was published. The first *Leishmania* parasite whose genome was sequenced was *L. major* having 36 chromosomes. The sequencing result of *L. major* genome revealed 911 RNA genes, 39 pseudogenes, and 8272 protein-coding genes (Ivens et al., 2005). The chromosomes of *Leishmania* display an unusual pattern of genes distribution where the genes are arranged in long directional clusters. The genomes of *L. braziliensis* and *L. infantum* are also sequenced and show significant conservation of genes. The *L. braziliensis* and *L. infantum* genomes contain 8,314 and 8,195 genes respectively (Peacock et al., 2007). At present, genome sequences of many other *Leishmania* species are also available.

1.2. Leishmaniasis

Leishmaniasis is a neglected tropical disease caused by the different species of *Leishmania*. Leishmaniasis exists in three different clinical forms namely cutaneous, mucocutaneous and visceral leishmaniasis. Amongst all the visceral leishmaniasis is the most severe form responsible for causing 100% mortality rate if left untreated (Alemayehu and Alemayehu, 2017). Leishmaniasis is prevalent in 88 countries, having 12 million infected cases around the globe with 0.2-0.4 million new cases of visceral and 0.7-1.2 million new cases of cutaneous leishmaniasis per year. More than 90% of world's visceral leishmaniasis cases are found in Bangladesh, India, Ethiopia, Brazil, South Sudan and Sudan. Cutaneous leishmaniasis is more widely distributed throughout the America, Mediterranean basin and Western Asia. In India, visceral leishmaniasis is most prevalent in Bihar, West Bengal, Assam and eastern Uttar Pradesh. Bihar has the highest number of visceral leishmaniasis cases (1,000,000) with 10,000 cases of drug-resistance against Pentamidine, Amphotericin and antileishmanial antimonials therapies (Boelaert et al., 2009; Singh et al., 2010).

More than 75% of the global cutaneous leishmaniasis cases are found in Afghanistan, Colombia, Brazil, Algeria, Ethiopia, Costa Rica, Peru, North Sudan, Iran and Syria

(Georgiadou et al., 2015). *Leishmania* can cause co-infections with other pathogens like human immunodeficiency virus (HIV), *Mycobacterium tuberculosis*, *Trypanosoma cruzi*, *Salmonella* and *Schistosoma* (Bañuls et al., 2007). Particular *Leishmania*-HIV coinfections are most common and found in 35 endemic countries. The global distributions map of leishmaniasis is shown in Figure 1.3.

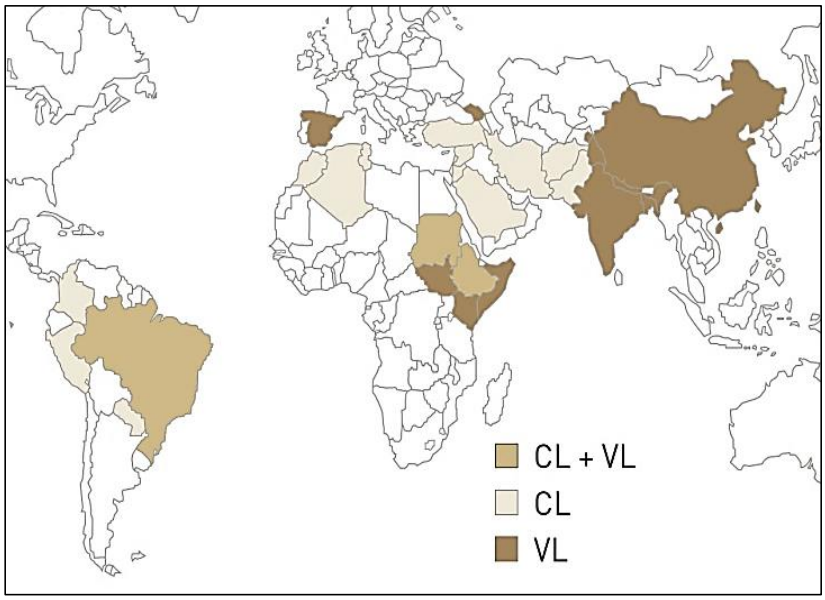


Figure 1.3: Geographical distributions of leishmaniasis. CL represents cutaneous leishmaniasis and VL represents visceral leishmaniasis. (Image source: https://www.dndi.org/diseases-projects/leishmaniasis/).

1.2.1. Cutaneous leishmaniasis

Cutaneous leishmaniasis is a less severe type of leishmaniasis consists of skin lesions (Figure 1.4 A). These skin lesions are generally self-healing but leave scars if untreated (Aoun and Bouratbine, 2014). Cutaneous leishmaniasis is caused by the *L. mexicana*, *L. tropica, L. braziliensis* and *L. major* complexes. It is prevalent in more than 80 countries (Cobo, 2014).

A variant clinical form of cutaneous leishmaniasis is known as diffuse cutaneous leishmaniasis which is caused by *L. Mexicana, L. aethiopica* and *L. amazonensis*. It is mainly found in South and Central America, Kenya and Ethiopia. Diffuse cutaneous

leishmaniasis consists of non-ulcerative nodules or plaques (Figure 1.4 B) typically on the face and extensor surfaces of the limbs.

1.2.2. Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis shows the lesions on mucous membranes of the nose, mouth, throat cavities and surrounding tissues (Figure 1.4 C). Mucocutaneous leishmaniasis is prevalent in Brazil, Paraguay, Ecaudor, Bolivia, Peru, Colombia and Venezuela. The pathogenesis of mucocutaneous leishmaniasis is not known. The development of mucocutaneous leishmaniasis is similar to that of cutaneous leishmaniasis, and both the infections can occur simultaneously. Mucocutaneous leishmaniasis occurs when cutaneous lesions spread to the mucosal region or through metastasis. The causative agents of mucocutaneous leishmaniasis include *L. braziliensis, L. major, L. tropica* and *L. infantum* (Cobo, 2014).

1.2.3. Visceral leishmaniasis

It is a most severe form of leishmaniasis caused by *L. donovani, L. infantum* and *L. chagasi.* It is prevalent in 47 countries and responsible for an estimated 500,000 infections each year worldwide. More than 90% of visceral leishmaniasis cases are found in India, Nepal, Bangladesh, Brazil and Sudan. It infects internal organs of the body (such as spleen, liver, and bone marrow), and can leads to the death if not treated. Signs and symptoms of visceral leishmaniasis include fever, headache, weight loss, lymphadenopathy, swelling of the liver and spleen (Figure 1.4 D), chronic diarrhea, darkening of the skin, fatigue, mucosal ulcers, anemia, coughing, and sometimes chronic kidney disease (Cobo, 2014). Visceral leishmaniasis may develop post-recovery symptoms, which include non-ulcerative cutaneous lesions. The condition is named as post kala azar dermal leishmaniasis (PKDL) (Figure 1.4 E). It mostly occurs after 1 to 2 years of completion of sodium antimony gluconate treatment. PKDL treatments are costly and time consuming (Zijlstra et al., 2003).

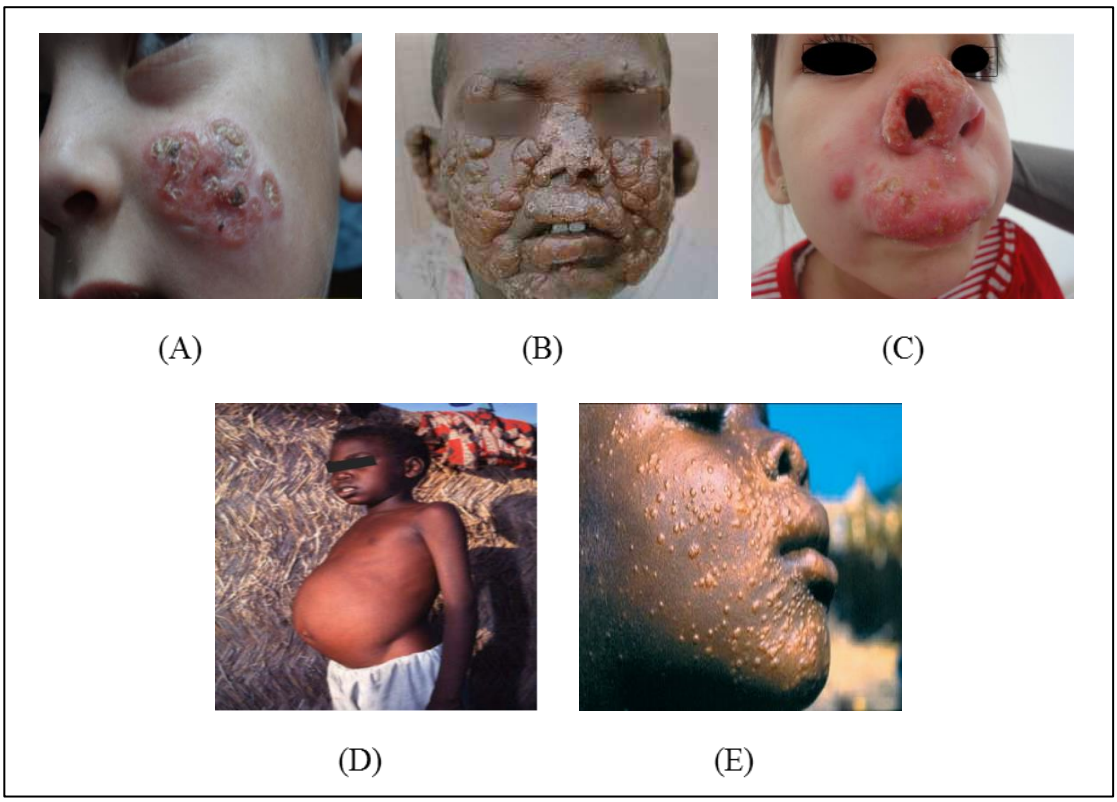


Figure 1.4: Types of leishmaniasis. (A) Cutaneous leishmaniasis (Image source: https://dermatologyoasis.net/cutaneous-leishmaniasis-3); (B) Diffuse cutaneous leishmaniasis (Source: http://animalia-life.club/other/diffuse-cutaneous-leishmaniasis.html); (C) Mucocutaneous leishmaniasis (Source: Elfaituri et al., 2015); (D) Visceral leishmaniasis (Source: https://www.deduveinstitute.be/~opperd/parasites/leish4.htm); (E) Post kala azar dermal leishmaniasis (Source: Zijlstra et al., 2003).

1.2.4. Disease transmission vectors

Leishmaniasis is transmitted by the sandfly vectors of genus *Phlebotomus* in the old world and *Lutzomyia* in the new world. The transmission occurs through the bite of sandfly during blood feeding. The sandfly saliva carries certain anticoagulating and vasodilatory components which promote blood flow and certain immunomodulatory elements which inhibit wound healing and suppresses the immune response (Secundino et al., 2012). Sandfly is more active during the twilight, evening and nigh-time, however, remain less active during the daytime especially when temperatures are high. Sandfly

usually lays its eggs in the burrows of certain rodents, ruined buildings, in the bark of old trees, in cracks of walls, in animal shelters and in house hold garbage since sandfly larvae need organic matter and moist and warm temperatures for their growth. Generally, the female sandflies are responsible for the transmission of the parasites since it can travel a distance of several hundred meters in the search for blood for the development of their eggs. Out of the 500 phlebotomine species, about 30 species can transmit leishmaniasis (Sharma and Singh, 2008). These include *Platanus argentipes* in Indian sub-continents, *P. martini* and *P. orientalis* in Africa and in Mediterranean basin, *P. chinensis* and *P. alexandri* in China. The *Lutzomyia logipalpis* is the main vector of *L. donovani chagasi* in the new world (Lainson and Shaw, 1972).

1.2.5. Diagnosis and treatments

The clinical signs and symptoms of leishmaniasis are not enough to get an accurate diagnosis of the disease. Hence various laboratory methods are required for the diagnosis of leishmaniasis. The most common method is the microscopic examination of amastigotes in the infected tissues samples such as bone marrow, spleen, lymph nodes, liver and skin. The other methods include serological and molecular methods which are more sensitive and specific than direct methods of diagnosis. The commonly used serological methods are ELISA, DAT and IFAT in which the parasite antigens are detected in blood, urine or tissue samples using antileishmanial antibodies (Portus et al., 1995; Vinayak et al., 1994). Molecular methods work by detection of parasite DNA in tissue samples (Cangalya et al., 1993). Other known methods of diagnosis are indirect haemagglutination, counter-current immunoelectrophoresis, immunodiffusion etc. However, due to the lack of sensitivity and specificity, these methods are rarely used (Singh, 2006).

Current treatment of leishmaniasis includes pentavalent antimonials that existed for 70 years. Pentavalent antimonials have a 60% failure rate and a 3–5% deaths rate (Croft et al., 2006). Other drugs include Miltefosine, Amphotericin B, Pentamidine and Paromomycin. Miltefosine is used as first-line therapy for visceral leishmaniasis but it is teratogenic and it rapidly gives resistance due to long half-life. Amphotericin B is used in two forms, Conventional and Liposomal form. The conventional form can cause life-threatening side effects and it also takes a longer time than liposomal form. The

liposomal forms are more effective and have lesser side effects but it is very expensive and complex in administration. The Ambisome is also found highly effective in India. The use of Pentamidine has been reduced due to the appearance of resistance cases, high toxicity, and low efficacy. Paromomycin also has adverse side effects like nephrotoxicity, hepatotoxicity and ototoxicity (de Menezes et al., 2015). The list of existing leishmanial drugs is given in Table 1.1.

Drug	**Mode of administra-tion**	**Type of leishmaniasis**	**Side effects**
Pentavalent antimonials	Intravenous or intramuscular	VL and CL	Pain, erythema, edema, abdominal pain, nausea and thrombocytopenia
Amphotericin B fungizone	Intravenous	For VL, CL and MCL	Infusion related, azotemia, anemia, or hypokalemia
Amphotericin B ambisome (Unilamellar liposome)	Intravenous	VL, PKDL and MCL	Hypotension, anorexia, nausea, vomiting, headache and weakness
Miltefosine	Oral	VL and some forms of CL	Nausea, vomiting and/or diarrhea, increased creatinine level
Amphotericin B formulation (Lipidic formulation)	Intravenous	VL	Shaking chills, nausea, hypotension, anorexia, headache and vomiting
Paromomycin	Intramuscular for VL and topical for CL	VL and CL	Pain, erythema, edema, blisters, and ototoxicity
Pentamidine	Intramuscular	For specific forms of CL	Nausea, vomiting, diarrhea, hyperglycemia, and cardiotoxicity

Sitamaquine	Orally active	VL	Abdominal pain, headache, vomiting, dyspepsia and cyanosis

Table 1.1: List of existing drugs of leishmaniasis (Bhargava and Singh, 2012).

1.3. Identification of drug targets in *Leishmania*

High toxicity, high cost and the development of drug resistance are major problems associated with the existing antileishmanial therapies, suggesting the need for identifying and understanding the new class of the drugs. The critical step in the process of drug designing is to identify the potential drug targets in the biological pathways which are essential for the survival and growth of the parasite and are either absent in the host or significantly different from the host homologs. The genome sequences of parasites can be utilized to identify the novel drug targets against the parasites.

In order to develop the candidate drug molecule, a multi-disciplinary approach has to be adopted starting from the characterization of the target protein to high throughput screening of compounds. Several bioinformatics approaches help to fasten the process. While selecting the target proteins, its biochemical properties and expression profile at different stages of the life-cycle of the pathogen has to be considered (Chawla and Madhubala, 2010).

1.4. Potential drug targets in *Leishmania*

Search for new potential drug targets mainly focus on biochemical and metabolic pathways that are crucial for the survival and growth of the parasite. *Leishmania* has a diverse set of biochemical and enzymatic machineries that substantially differ from their hosts. Some of the pathways such as Carbohydrate metabolism (Glycolysis pathway), Sterol biosynthetic pathway, Purine salvage pathway, GPI biosynthetic pathway, Folate biosynthesis, Glyoxalase system, Trypanothione pathway and Hypusine pathway show the uniqueness in terms of structural and biochemical difference within host and parasite makes them as a potential drug target. Many of these pathways are compartmentalized in glycosomes. Several classes of proteins like Topoisomerases, Protein kinases, Proteinases, MAP kinases have also been identified as drug targets (Chawla and

Madhubala, 2010; Singh et al., 2012). Purine salvage pathway and Glycolytic pathway are important for the survival and growth of *Leishmania* and therefore have been of interest for this study.

1.4.1. Purine salvage pathway

The most striking disparities between human and *Leishmania* metabolic machinery are the method of synthesis of purine nucleotides. There are two pathways for the synthesis of purine bases. The one in which the purine nucleotides are build on 5-phosphoribosyl-alpha-1-pyrophosphate (PRPP) is called *de novo* biosynthesis pathway and the other pathway is called salvage pathway, where the purine bases are recovered from the degradation products of nucleotide metabolism.

Humans can synthesize purine nucleotides by *de novo* pathway whereas *Leishmania* lacks the metabolic machinery for *de novo* synthesis and acquire purine bases from the host via salvage pathway (Hammond and Gutteridge, 1984). The host purine bases are internalized in the parasite cell through nucleoside transporters. *Leishmania* has two known cell surface nucleoside transporters namely LdNT1 and LdNT2. The LdNT1 can transport adenosine and pyrimidine nucleosides and they are found in both promastigote and amastigotes stages. Whereas LdNT2 can transport purine nucleosides (inosine and guanosine) and are found in amastigotes (Carter et al., 2000; Vasudevan et al., 1998). Other nucleobase transporters are LmaNT3 which transports hypoxanthine and xanthine and LmaNT4 which can transport adenine at neutral pH and hypoxanthine, guanine and xanthine at acidic pH (Ortiz et al., 2009). LmaNT4 is important for the viability of the parasite inside the acidic phagolysosomes of human macrophages. Although nucleoside transporters are important for the purines metabolism of the parasite, it is difficult to target them with selective inhibitors because there are number of different pathways available for the uptake of purines (Chawla and Madhubala, 2010).

The host purines can be metabolized into different products. For example, an adenine can be converted to hypoxanthine by enzymes adenine deaminase which is further phosphoribosylated to IMP (Inosine monophosphate) by hypoxanthine phosphoribosyl transferase. Alternatively, adenine can be converted to AMP (Adenosine monophosphate) by adenine phosphoribosyl transferase. The AMP can be converted to IMP or ADP

(Adenosine diphosphate) by AMP deaminase and AMP kinase. Similarly, guanine and other bases can be converted to their respective nucleotide monophosphates. The purine salvage pathway of *Leishmania* species is shown in Figure 1.5.

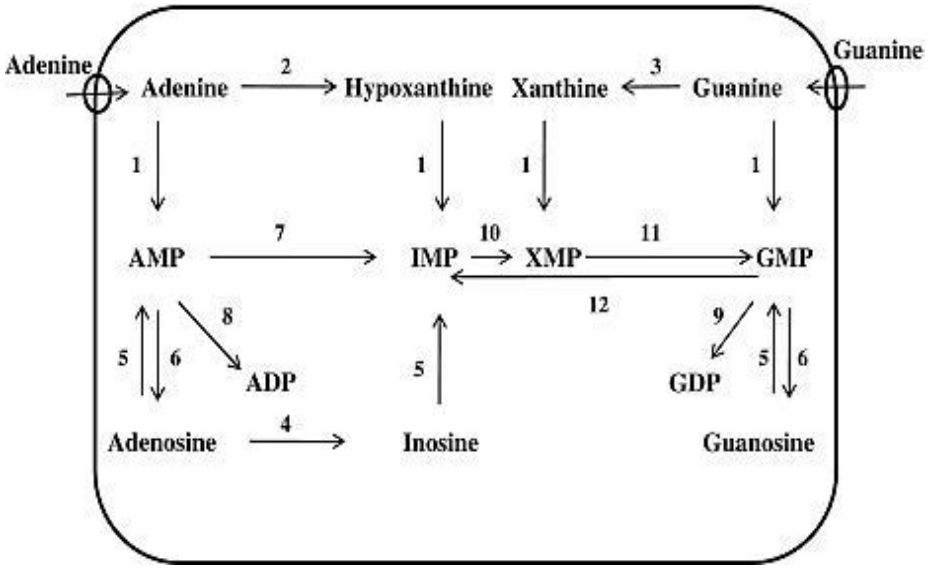


Figure 1.5: Purine salvage pathway of *Leishmania spp.* The enzymes involved in salvage of purines are: 1) Phosphoribosyltransferase; (2) Adenine deaminase; (3) Guanine deaminase; (4) Adenosine deaminase; (5) Nucleoside kinase; (6) Nucleotidase; (7) AMP deaminase; (8) AMP kinase; (9) GMP kinase; (10) IMP dehydrogenase; (11) GMP synthetase; (12) GMP reductase. Image source: (Chawla and Madhubala, 2010).

1.4.1.1. Phosphoribosyl transferases

Phosphoribosyl transferases (PRTs) play crucial role in the purine salvage pathway. PRT enzymes carry out the transfer of ribosyl-5-phosphate group from 5-phosphoribosyl-1-pyrophosphate (PRPP) to nucleobases such as adenine, guanine, hypoxanthine, xanthine etc. with the release of PPi. PRT family is classified as Type I and II based on their structural and catalytic features. The type I PRTs have a core domain of minimum five parallel β-strands surrounded by three or more helices and a common α/β-fold at the PRPP binding motif and a flexible loop. The Type II PRTs contain a mixed α/β N-terminal domain and an α/β barrel like C-terminal domain (Monzani et al., 2007). PRT proteins include a homologous family of enzymes and regulatory protein of the nucleotide synthesis and salvage pathway. *Leishmania* contains three PRT enzymes viz.

adenine phosphoribosyl transferase (APRT), hypoxanthine guanine phosphoribosyl transferase (HGPRT) and xanthine phosphoribosyl transferase (XPRT) (Glew et al., 1988). APRT catalyzes the conversion of adenine to AMP (Figure 1.6). HGPRT converts hypoxanthine and guanine to IMP and GMP respectively (Figure 1.7) whereas XPRT catalyzes the conversion of xanthine to XMP (Figure 1.8). In *L. donovani*, APRT is localized in cytosol whereas HGPRT and XPRT are localized in glycosome (Zarella-Boitz et al., 2004).

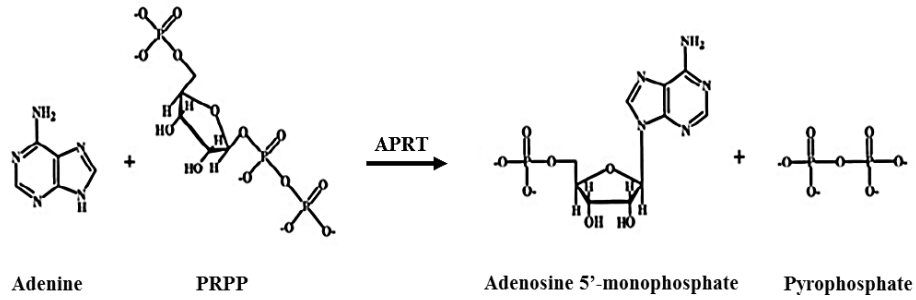
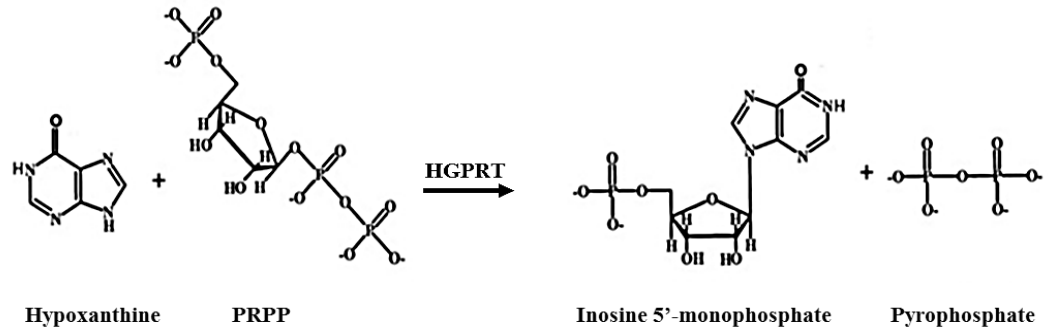
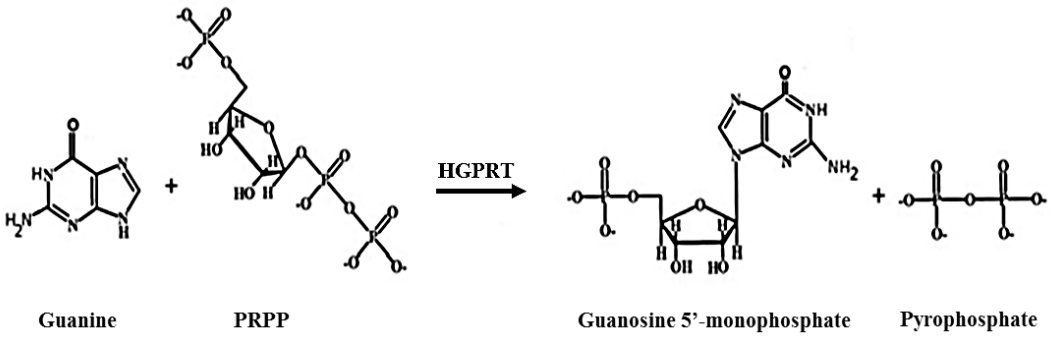


Figure 1.6: Reaction catalyzed by adenine phosphoribosyl transferase.



(A)



(B)

Figure 1.7: (A) & (B) Reactions catalyzed by hypoxanthine guanine phosphoribosyl transferase.

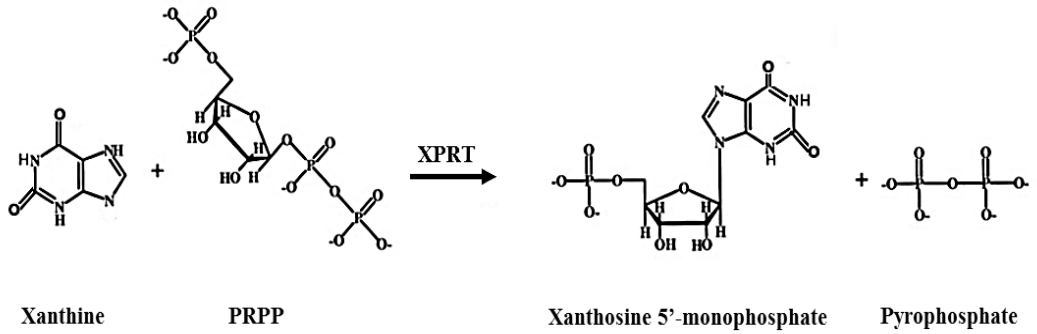


Figure 1.8: Reaction catalyzed by xanthine phosphoribosyl transferase.

The most studied PRT enzyme is HGPRT. There are 92 crystal structures of HGPRTs reported in the PDB database from 21 different organisms. The crystal structure of HGPRT from many different protozoans are solved including *L. tarentolae, T. cruzi, T. brucei, P. vivax, P. falciparum* and *T. foetus.* The *L. tarentolae* HGPRT structure has two chains (A and B), each with the bound GMP. The enzyme shows an intricate hydrogen bond network which is important for its activity and stability (Monzani et al., 2007). The crystal structure of *L. donovani* APRT is solved in complex with adenine, AMP, sulfate and citrate ions. The core region of *L. donovani* APRT is similar to that of other PRT enzymes, however, the adenine binding domain is quite different. Structurally *Leishmania* and human APRTs are different as human APRT lacks N (S1, S2) and C (S9) terminal strands and helices (H10-H12) (Phillips et al., 1996). The crystal structure of human HGPRT is also available with and without ligands. The structure of human HGPRT provides information about the active site which can be utilized for comparison with the parasite's enzyme active site for the designing of potential parasite-specific inhibitors.

In contrast to HGPRT, only two XPRT structures are solved. One is *B. subtilis* XPRT and the other is *E. coli* XPRT. The structure of leishmanial XPRT is not yet solved. The *B. subtilis* XPRT is known for its high specificity towards xanthine. The structure of *B. subtilis* XPRT revealed that its unique base specificity depends on the key residues of active site. It showed that Asn 27 provides an ability to bind both XMP and GMP with high affinity (Arent et al., 2006). Similarly, *E. coli* XPRT structure revealed that the

glutamine residue in the purine binding site might govern its 6-oxopurine base specificity (Vos et al., 1997).

Various inhibitors have been identified against *Leishmania* HGPRT enzyme e.g. allopurinol. Allopurinol is phosphorylated by HGPRT enzyme and incorporated into nucleic acids which further leads to the death of parasite (Fish et al., 1985; Marr, 1984). Allopurinol is effective against cutaneous (Martinez et al., 1997) and visceral leishmaniasis (Kager et al., 1981). Isatin and phthalic anhydride are competitive inhibitors of purine bases and capable of inhibiting parasitic HGPRT, without affecting the human HGPRT. Phthalic anhydride derivative -TF1 and phthalimide derivative -TF2, are capable of killing the cultured *Leishmania* (John R. Somoza et al., 1998). These findings suggest that the success of structure-based inhibitor designing approach can provide a useful starting point for drug design against parasitic diseases.

1.4.1.2. Xanthine phosphoribosyl transferase

The enzyme XPRT of *L. donovani* has been the focus of attention for a long time as it is absent in mammals. Xanthine phosphoribosylating enzymes in other organisms like *P. falciparum, T. gondii*, *T. foetus* and *E. tenella* contain a common hypoxanthine-guanine-xanthine PRT (HGXPRT) enzyme where xanthine is the least favored substrate as compared to hypoxanthine and guanine. However, *L. donovani* XPRT substrate profile is different from these enzymes, as it favors xanthine over hypoxanthine and guanine. The HGPRT enzymes cannot recognize xanthine, however, the XPRT can act on hypoxanthine and guanine in addition to xanthine. The previous report on null mutational studies of leishmanial PRTs suggested that the XPRT activity alone is sufficient to salvage adenine, hypoxanthine and guanine in *L. donovani* promastigotes (Jardim et al., 1999). Due to their unique substrate specificity compared to other PRT enzymes of the purine salvage pathway and lack of human counterpart, *L. donovani* XPRT is the potential target for parasite-specific drug discovery.

1.4.2. Carbohydrate metabolism pathways

Carbohydrate metabolism in *Leishmania* plays key role in the ATP supply and synthesis of glycoconjugates like LPG and are important for the viability and virulence of the human-pathogenic stages of the parasite. The most important carbohydrate is glucose,

which can be metabolized via glycolysis, Kreb's cycle and oxidative phosphorylation to generate ATP. In *Leishmania* parasite, the first biochemical pathway that was targeted for rational drug designing was the Glycolysis. All stages of *Leishmania* express the enzymes for glycolysis (Myler and Fasel, 2008). In contrast to human glycolytic enzymes, the major part of *Leishmania* glycolysis pathway is present in the Glycosome. The compartmentalization of the glycolytic pathway in glycosome results in a net balance of ATP and NADH generation and consumption (Harris, 2015). Various glycosome-localized enzymes of glycolysis (e.g. hexokinase) are involved in the synthesis of β-mannan, a mannose-rich polysaccharide that is essential for amastigote replication and thus virulence. *Leishmania* uses mannan as a primary energy reserve (Ralton et al., 2003) and its glycoprotein coat also has high mannose content (Davis et al., 2004; Harris, 2015). The glycolytic pathway of *Leishmania* is shown in Figure 1.9.

The unique localization of glycolytic enzymes in glycosomes and their large phylogenetic distance from mammalian hosts makes them important targets for structure-based drug designing in *Leishmania* (Chawla and Madhubala, 2010). In many cases, these differences have been utilized to design and synthesize parasite-specific inhibitors. Several glycolytic enzymes like glyceraldehyde-3-phosphate-dehydrogenase, glucose-6-phosphate isomerase and aldolase have been studied over the last three decades in terms of enzyme kinetics, enzyme crystallization and screening for inhibitors. The first known antileishmanial drug sodium stibogluconate was found to inhibit *L. mexicana* amastigote glycolysis (Berman et al., 1987). The most recent studies of targeting *Leishmania* glycolytic pathway were based on inhibition of *L. mexicana* pyruvate kinase by furanose sugar amino amides (Nowicki et al., 2008).

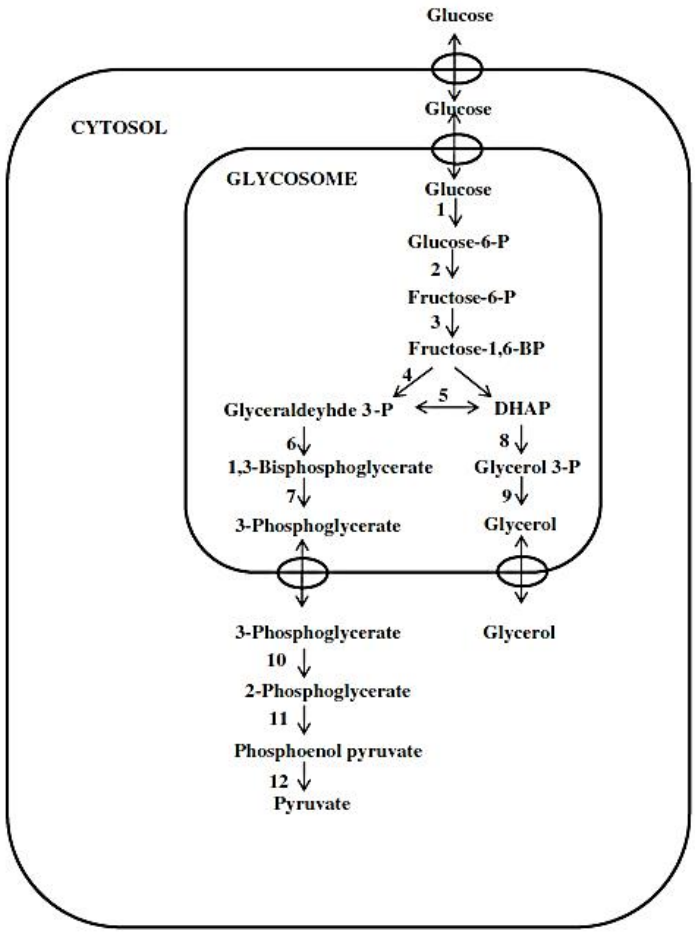


Figure 1.9: Glycolytic pathway in *Leishmania spp*. The enzymes involved in the pathway are (1) Hexokinase; (2) Glucose-6-phosphate isomerase; (3) Phosphofructokinase; (4) Aldolase; (5) Triosephosphate isomerase; (6) Glyceraldehyde-3-phosphate dehydrogenase; (7) Phosphoglycerate kinase; (8) Glycerol-3-phosphate dehydrogenase; (9) Glycerol kinase; (10) Phosphoglycerate mutase; (11) Enolase; (12) Pyruvate kinase. Image source: (Chawla and Madhubala, 2010).

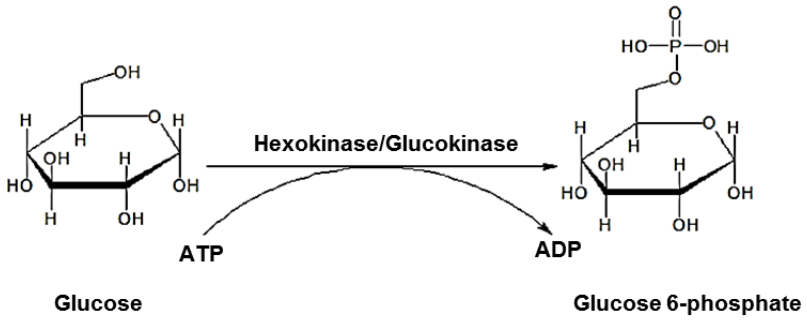
1.4.2.1. Hexokinase

The first step of glycolysis is catalyzed by hexokinase which is the phosphorylation of glucose into the glucose 6-phosphate. The glucose 6-phosphate is utilized in number of pathways such as in the synthesis of ATP or for the synthesis of NADPH or used as precursors of nucleic acids. Hence, the inhibition of hexokinase would directly interfere in the number of metabolic processes. This reaction is also important to trap glucose inside the cells in the form of glucose 6-phosphate, since glucose is highly permeable to

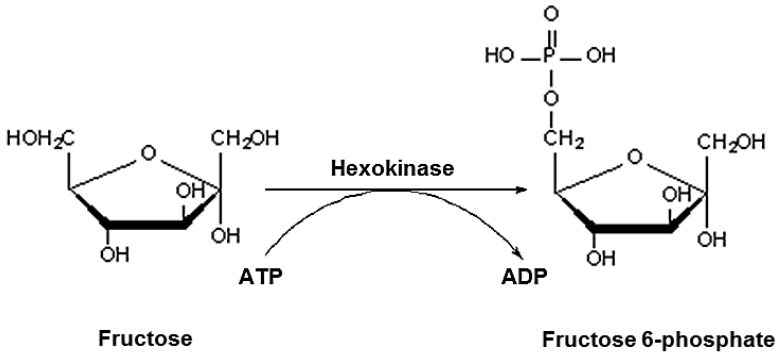
the plasma membrane, hence it allows cells to stock up glucose during high-glucose conditions. Hexokinase can catalyze the phosphorylation of a variety of hexose sugars including glucose, fructose (Figure 1.10) and mannose. Hexokinase also contributes to mannose metabolism by catalyzing the formation of glucose-6-phosphate which is a precursor of mannose-6-phosphate or by direct phosphorylation of mannose (Harris, 2015).

Hexokinase has PTS (Peroxisomal Targeting Signal) peptide on N- terminal. PTS targets protein towards peroxisome. Despite having a PTS, hexokinase may moonlight as a hemoglobin receptor in the flagellar pocket. *Leishmania* lacks the ability for *de novo* biosynthesis of heme hence the heme is acquired from the host's hemoglobin. Hence in addition to their catalytic function hexokinase also helps in internalization and subsequent intracellular degradation of hemoglobin to generate heme, which is crucial for their growth (Krishnamurthy et al., 2005). *Leishmania* genome has two nearly identical hexokinases in tandem on chromosome 21 with 89 to 99 percent amino acid identity (Harris, 2015).

Hexokinase from *L. mexicana* has been characterized. The enzyme shares kinetic characteristics with hexokinase of *T. cruzi*. Although in contrast with *T. cruzi* hexokinase, *L. mexicana* enzyme exhibit moderate inhibitory activity by glucose 6-phosphate (Pabón et al., 2007). There are no crystal structures of hexokinase reported from protozoans till date. However, the modeled structure of the *T. brucei* hexokinase-inhibitor complex has been reported. The inhibition studies of the *T. brucei* hexokinase, have provided valuable information about the mode of action of glucosamine analog inhibitors (Willson et al., 2002). Several bisphosphonates were identified as effective inhibitors against the native hexokinase enzyme at sub-micromolar levels and some are also active against the amastigote form of *T. cruzi* (Hudock et al., 2006).



(A)



(B)

Figure 1.10: (A) & (B) Reactions catalyzed by hexokinase and glucokinase.

1.4.2.2. Glucokinase

The hexokinase family comprises three groups: hexokinase group, A group and B group. Group A consists ATP-dependent glucokinases that are generally found in Cyanobacteria, Gram-negative bacteria and mitochondriate protists, whereas group B contains ATP-dependent hexokinases found in Crenarchaeota and glucokinases of Gram-positive bacteria which either use ATP or polyphosphate as phosphoryl donor. Unlike hexokinase, glucokinase can catalyze the phosphorylation of only D-glucose. Glucokinase has a low affinity for glucose, suggesting it may provide glucose-6-phosphate for non-catabolic processes like the synthesis of the *Leishmania* virulence factor mannan or surface-bound glycoconjugates like LPG, or for the pentose-phosphate pathway under high-glucose conditions while hexokinase activity is controlled through PPi by the cellular energy charge (Cáceres et al., 2007).

The structure of *T. cruzi* glucokinase, in complex with glucose and ADP, revealed that in contrast to hexokinases, which show a moderate preference for the α-anomer of glucose, *T. cruzi* glucokinase was bound with the β configuration of glucose. The preference of *T. cruzi* glucokinase for β-D-glucose was also confirmed by kinetic assay. Structural comparison of the glucokinase and hexokinase may help to identify the possible mechanism of their anomer selectivity (Cordeiro et al., 2007). The structure of glucokinase from *L. braziliensis* has been determined with 1.85 Å resolution in apo form. The enzyme is a homodimer where each subunit includes the typical small and large domains. The inhibition studies of *L. braziliensis* glucokinase with glucosamine analog inhibitors, identified HPOP-GlcN (hydroxyphenyloxopropyl glucosamine) as the strongest inhibitor (Buechner et al., 2019). The glucosamine analogs Carboxybenzyl glucosamine and benzoyl glucosamine are known as inhibitors for *T. cruzi* glucokinase (D'Antonio et al., 2015).

1.5. Rationale behind the current study

Leishmaniasis remains a major health problem today. The treatment is difficult, because of the presence of three different forms and various clinical manifestations. High toxicity, high cost and the development of drug resistance are major problems of existing antileishmanial drugs. There are only a few drugs available for the treatment of leishmaniasis and hardly anything is known about their mode of action. Hence, there is a need to identify and characterize the new prospective drug targets and to develop parasite-specific inhibitors. A rational approach to develop new drugs deals with identifying metabolic differences between the host and parasite and exploit those proteins which are essential for the survival of parasite and are not important for the host. The structure-based approach helps to identify the key differences between the structures of the human and parasite proteins that can be exploited to develop more specific and effective drugs. Thus, in the present study, we have selected three such enzymes (xanthine phosphoribosyl transferase, hexokinase and glucokinase) from the purine salvage pathway and carbohydrate metabolism pathway of *L. donovani*, based on their importance in parasite pathogenesis and survival in the host.

The overall aim of this study is to characterize the structural and functional aspects of these proteins for selective inhibition to exploit them for rational drug designing. In this

work we are attempting to crystallize the target proteins to solve their three-dimensional structure and identify the potential inhibitors for these proteins using the multidisciplinary approach right from the computational screening of compounds to identify the affinity and inhibitory activities of the compounds by biochemical & biophysical approach.

1.6. Aims and objectives of the present study

The major objectives for the present study are as follows.

1. Cloning, expression and purification of target proteins (xanthine phosphoribosyl transferase, hexokinase and glucokinase) from purine salvage pathway and carbohydrate metabolism pathways of *Leishmania donovani*.
2. Functional characterization of target proteins.
3. Biophysical characterization of target proteins.
4. Structural characterization of target proteins using Homology modeling and X-ray crystallography.
5. Screening of inhibitors for target proteins by computational docking approach and by enzyme inhibition assay.

Chapter 5

Summary and Conclusion

The critical dependence of *Leishmania* on purine salvage enzymes and glycolytic enzymes offers an opportunity to target them for chemotherapeutic intervention. Among all PRT enzymes of the purine salvage pathway in *Leishmania*, XPRT is unique in its substrate specificity and their non-existence in human. It is an interesting protein not only for drug designing but also to understand the molecular determinants of its substrate specificity. Enzymes hexokinase and glucokinase are also potential targets to develop parasite-specific inhibitors as they catalyze the phosphorylation of glucose to glucose 6-phosphate, which is the precursor for number of metabolic processes.

In Chapter 2, a detailed study was done on heterologously expressed LdXPRT protein from *Leishmania donovani*. The recombinant LdXPRT protein was in a monomeric state with a molecular weight of 27 kDa. To date, only *E. coli* and *B. subtilis* XPRT enzyme's structures are solved. In order to get the structure of LdXPRT, crystallization trials were carried out. Most of the crystals conditions gave salt crystals while few were not suitable for diffraction by X-ray beam hence computational approach was applied to obtain the structure of LdXPRT. The structure of LdXPRT was generated by homology modeling. The model was refined further by energy minimization and the accuracy of the model was within the domicile of reported X-ray crystal structures to date. The LdXPRT model is an α/β type protein carrying the PRTase type I folding pattern which has a characteristic core domain consisting of phosphate binding site and the hood domain consisting of purine binding site. The PRPP loop (153-161) which binds to the ribose 5-phosphate group of PRPP was present between β6 and α6 and the PPi loop (92-95) which binds to pyrophosphate group of PRPP was present between β2 and α4. The flexible loop (118 to 147) which is involved in the formation of a transition state was found between β3 and β6. Two acidic residues Glu 153, Asp 154 whose side chains form hydrogen bonds with the ribose hydroxyl groups were conserved in LdXPRT and were located at the beginning of the PRPP loop. The dipeptide SY which helps the flexible loop to adopt a closed conformation upon binding with the PRPP was conserved in LdXPRT. In addition to dipeptide SY, the residues Ser 129, Thr 130, Gly 131 and Ile 133 were also conserved in this region. The presence of an additional Glycine (Gly 126) in LdXPRT

flexible loop imparted more conformational flexibility allowing the polypeptide to take a turn which resulted in many interactions between residues within the loop.

Comparison of LdXPRT model with the human counterpart reveals notable differences between the two which can be considered for future drug development. Active site residues of human HGPRT-GMP complexed structure showed three major interactions with the purine ring of guanine base. The interaction between Lys 165 and 6-oxo group of guanine, the aromatic stacking interaction between Phe 186 and the purine ring and the interaction between the Asp 193 and Val 187 with the 2-amino group of the guanine base. The Lys was conserved in LdXPRT (Lys 186) however it had an Isoleucine (Ile 209) in place of Val 187 and a Glutamate (Glu 215) in place of Asp 193. The aromatic stacking interaction might be caused by Tyr 208. Most of the PRT enzymes that act on the 6-oxopurines like the leishmanial HGPRT and human HGPRT can act on hypoxanthine and/or guanine as substrates but can not utilize xanthine. The enzyme assay showed that the LdXPRT can recognize hypoxanthine and guanine, in addition to its natural substrate xanthine.

To identify the potential inhibitors for LdXPRT, the multidisciplinary approach was used right from the computational screening of compounds to identify the affinity and inhibitory activities of the compounds by biochemical & biophysical approach. The compound libraries of substrate analogs, existing antileishmanial drugs and natural compounds were screened against LdXPRT by virtual screening. Few compounds from docking studies of LdXPRT were characterized further for their affinity and inhibitory potential by fluorescence spectroscopy and enzyme inhibition assay. The highest inhibitory activity was shown by dGMP (61.36%) and cGMP (51.14%). Four (dGMP, cGMP, dGDP & GTP) compounds showed competitive inhibition with both the substrates (xanthine & PRPP) of LdXPRT. The enzyme inhibition assay revealed that there was an inverse relationship between the number of phosphate groups and the inhibition activity of XMP analog compounds. Binding of inhibitors causes a minor shift in the secondary structure of LdXPRT with a noticeable change in tertiary structure pattern whereas the binding of natural substrate xanthine and PRPP cause a minor effect on the tertiary structure of the protein. Binding of xanthine and PRPP separately did

affect secondary structure but both when added concurrently show a higher change in the spectra, which is an indication of the conformational change due to the product formation.

The distribution of XPRT in phylogeny tree was very scattered and found only in a few organisms like *Leishmania spp.* and certain fungi and bacteria. XPRT gene was not detected in other protozoan parasites and humans. Instead, the protozoa, which do not have XPRT gene have evolved their HGPRT in the form of HGXPRT to utilize xanthine in addition to its regular substrates, hypoxanthine and guanine e.g. *Toxoplasma gondii* HGXPRT (Heroux et al., 1999). *Leishmania* typically has both (XPRT and HGPRT) these 6-oxopurine PRTs. An intriguing feature of PRT enzymes are the molecular switches that control their unique 6-oxopuine (hypoxanthine, guanine & xanthine) specificities (Ullman et al., 2010). In order to determine the residues governing the 6-oxopurine specificity of these enzymes, the active site of LdXPRT and *L. tarentolae* HGPRT enzyme were compared. Sequence and structure comparison identified three probable amino acids (Ile 209, Glu 215 and Tyr 208) that may constitute the basis for purine base specificity of the LdXPRT. In chapter 3, the mutation analysis of these amino acids was carried out to probe their role is substrate specificity. The LdXPRT sequence contains a unique motif (55 to 82) which was absent in all PRTs enzymes including leishmanial HGPRTs. In MD simulations this region showed higher fluctuation upon binding of substrate. Residues of this region also showed local interactions with the core domain near to which the PRPP and PPi binding loops are located. Hence it was presumed that this region may have a conformational impact on PRPP and PPi binding loop during substrate binding. Hence to investigate the role of this region in XPRT activity and/or specificity, this region was deleted from LdXPRT for further characterization.

Based on *in silico* analysis, four mutants were generated viz. ldxprtY208F, ldxprtI209V, ldxprtI209V;E215D and ldxprtL55_Y82del. All the mutations were introduced in LdXPRT gene by a polymerase chain reaction. The mutants were expressed in *E. coli* Rosetta (DE3) cells and purified through Ni-NTA affinity chromatography. All purified

proteins were in stable and folded form. The secondary and tertiary structure pattern of LdXPRT and ldxprtY208F were almost similar although other mutants have some differences from the wild type protein. The I209V mutation induced a significant change in tertiary structure pattern but when I209V was mutated together with the E215D there was only slight change in tertiary structure indicating that the combination of Val and Glu alters the environment of phenylalanine and tyrosine residues whereas the combination of Val and Asp does not affect the structure. The proteins (LdXPRT & ldxprtY208F) having higher activity for XMP showed more helical content than the proteins (ldxprtI209V, ldxprtI209V;E215D & ldxprtL55_Y82del) having lower affinity for XMP. The proteins (wild type protein, ldxprtY208F & ldxprtL55_Y82del) having Ile at position 209 gave negative peak at 257 nm upon binding with GMP whereas the proteins (ldxprtI209V, ldxprtI209V;E215D) in which I209 was mutated to Val, gave positive peak upon GMP binding, indicating that the interaction of GMP to Ile 209 might be responsible for this change. Binding of XMP also causes a significant change in tertiary structure pattern of the native protein.

The fluorescence spectra of ldxprtI209V showed a significant change from native protein as indicated by the shift in $\lambda_{max.}$ It is probably due to the change in protein structure which further cause a change in the exposure of aromatic residues. Similar to CD spectra, fluorescence spectra of LdxprtI209V;E215D showed a similar pattern as that of the native protein. Deletion of L55_Y82 also causes a shift in λ_{max} from 305 nm to 337 nm. The results of fluorescence quenching were in agreement with the enzymes assay where wild type enzyme and ldxprtY208F showed a higher affinity for XMP than IMP and GMP. However, ldxprtI209V and LdxprtI209V;E215D showed a higher affinity for IMP and GMP.

LdxprtI209V show a significant decrease in affinity for xanthine in enzyme assay. Since almost all the HGPRT enzymes have Val in place of Ile 209 and they cannot recognize xanthine, it can be presumed that Val might interfere in the binding of xanthine. It was also observed in *T. gondi* and *T. foetus* HGXPRTs which contain Ile in the binding site have good affinity for xanthine whereas *P. falciparum* HGXPRT have a very low affinity

with xanthine since it has Val in place of Ile. Interestingly PRTs having Gln in place of Val (e.g. *S. faecalis* and *L. casei*) also have a higher affinity for xanthine. Hence the probable reason for substrate specificity of LdXPRT might be the presence of Ile at position 209 in the purine binding pocket. The I209V;E215D mutation causes further increase in affinity of hypoxanthine and guanine. Hence I209V and I209V;E215D mutations converted ldXPRT to HGXPRT like enzyme which exhibits nucleobase specificity comparable to *T. gondi* (Heroux et al., 1999), *T. foetus* (Munagala and Wang, 1998) and *P. falciparum* (Roy et al., 2015) HGXPRT. LdxprtY208F does not give significant change in specificity as it also chose xanthine over hypoxanthine and guanine. Mutation E215D cause a marked increase in turnover rates with all purine bases. Deletion of unique motif L55_Y82del ceased the activity of the enzyme indicates that the region is either important for enzyme activity or it might be altering the enzyme's structure in such a way that it could not perform enzyme reaction.

In chapter 4, the LdHK and LdGK genes were cloned and expressed in different *E. coli* expression strains. As LdHK remained insoluble even after different expression trials, we did not proceed with its purification. LdGK was purified successfully having a molecular weight of 44 kDa. LdGK presented the close ancestral association with the glucokinases from *Trypanosoma and Trichomonas vaginalis* however, it was evolutionarily very distant from human glucokinase. Glucokinase may occur in different oligomeric forms depending on their concentration. The purified LdGK used in the present study was in the monomeric state at 3 mg/mL concentration in the given buffer conditions. The purified recombinant LdGK showed 31.8% of α-helix, 24.8% of β-sheet and 12.8% turns. The crystallization was done using varying conditions of temperature, pH, and buffer to get the structure of LdGK. Crystallization trials gave few probable protein crystals but they got broken upon exposure with the X-ray beam. These conditions are being optimized further to get better quality crystals. The LdGK model was generated by homology modeling and the structure was used for further studies. Comparison of LdGK model with the human counterpart reveals significant differences between the two. The active site comparison of LdGK with human glucokinase identified two major differences. In human glucokinase-glucose structure, residues Asn 231 and Thr 168 are involved in the

interaction with O4 and O2 atoms of glucose respectively. LdGK did not have these residues, instead, it contains Gly 201 in place of Asn 231 and Asn 117 corresponding to the residue Thr 168. The compound libraries of glucose analogs and natural compounds were screened against LdGK by virtual screening. Some of these compounds showed more than -12 kcal/mol of binding affinity which was much higher than that of its natural substrate. Further optimization of these compounds can give potential drug molecules against leishmaniasis.